Figure 1 A

SEQ ID NO: 1

/translation="MGSVLSTDSGKSAPASATARALERRRDPELPVTSFDCAVCLEVL

HQPVRTRCGHVFCRSCIATSLKNNKWTCPYCRAYLPSEGVPATDVAKRMKSEY
KNCAE

CDTLVCLSEMRAHIRTCQKYIDKYGPLQELEETAARCVCPFCQRELYEDSLLDHC
ITH

HRSERRPVFCPLCRLIPDENPSSFSGNLIRHLQVSHTLFYDDFIDFNIIEEALIRRVL
DRSLLEYVNHSNTT"

Figure 1 B

SEQ ID NO: 2

AGCGGAGGTCATTTTTGCAGCTTATTGTGATGACAACAGTGGAGGATGGTC
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TTCAATCCTCCCAATACCACAAGACAGAGCACGGGGCGGCTGCCGCCTCC
GCCTCCGCGCCTTAACCTAGGCGGCTTGCCGAAGATCTCAGCCCCGCGGC
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GGTGTGTATGTCCCTTTTGTCAGAGGGAACTGTATGAAGACAGCTTGCTGG
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1861 acactccagc ctgggtaaca gagctagact ccatctcaaa aaaaaaaaaa aaaaaa
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127   gcgg ctgccgcctc cgcctccgcg ccttaaccta ggcggcttgc cgaagatctc
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Figure 1 E

TRAC1 genomic region:

SEQ ID NO:5

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SEQ NO:5
cont'd

Figure 1 E cont'd

SEQ NO:5 cont'd

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AAGTCCATTTAGGCCGGACGCGGTGGCTTACGCTTGTAATCCCAGCACTT
TGGGAGGCTGAAGCAGGCGGATCACAAGGTCAGGAGTTCGAGACCAGCCT
GGCCAACACTGGTCTCTGTGAAACTCCGTCTCT

Figure 1F

Mouse TRAC1 cDNA sequence:

```
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CGAGCCAGGAGGCGGCGATCCGGCCTGGGCCGGGACTTCCTTCCTCCCGG
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TGAGCTAGAGCGCAAGNNTGGGCTCCCTGCTGAGCAGCGACAGCTCCAAG
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```

Mouse TRAC1 protein (3rd frame)

SAXXGSLLSSDSSKSAPASATPRTLERSGDSELPITSFDCSVCLEVLHQP

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ELDEDCLLDHCIIHHRSERRPVFCPLCHSRPDESPSTFNGSLIRHLQVSH
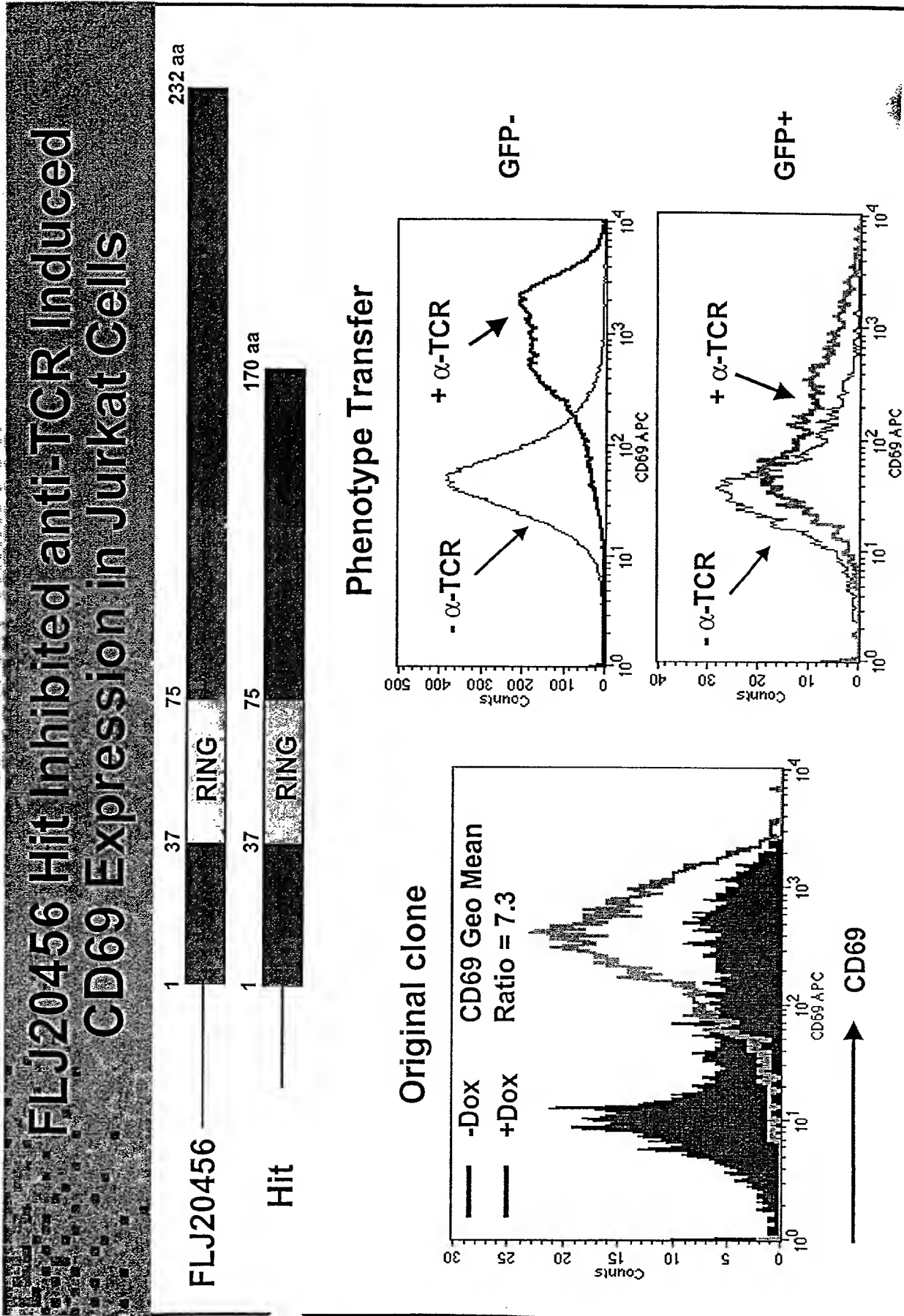
TLFYDDFIDFDIIEEAIIRRVLDRSLLEYVNQSNTTFYD

Figure 2

FLJ20456 Hit Inhibited anti-TCR Induced CD69 Expression in Jurkat Cells

FLJ20456

Hit

Phenotype Transfer

GFP-

GFP+

Original clone

CD69 Geo Mean
Ratio = 7.3

-Dox
+Dox

CD69

Figure 3

Full Length FLJ20456 Does Not Inhibit CD69 Upregulation in Jurkat Cells

FLJ20456

1   37   75   RING   232 aa

- Pfu PCR product amplified from a capped human brain cDNA library.
- One N to S polymorphism with FLJ20456 NM_017831.1 at amino acid 186, present in EST database.
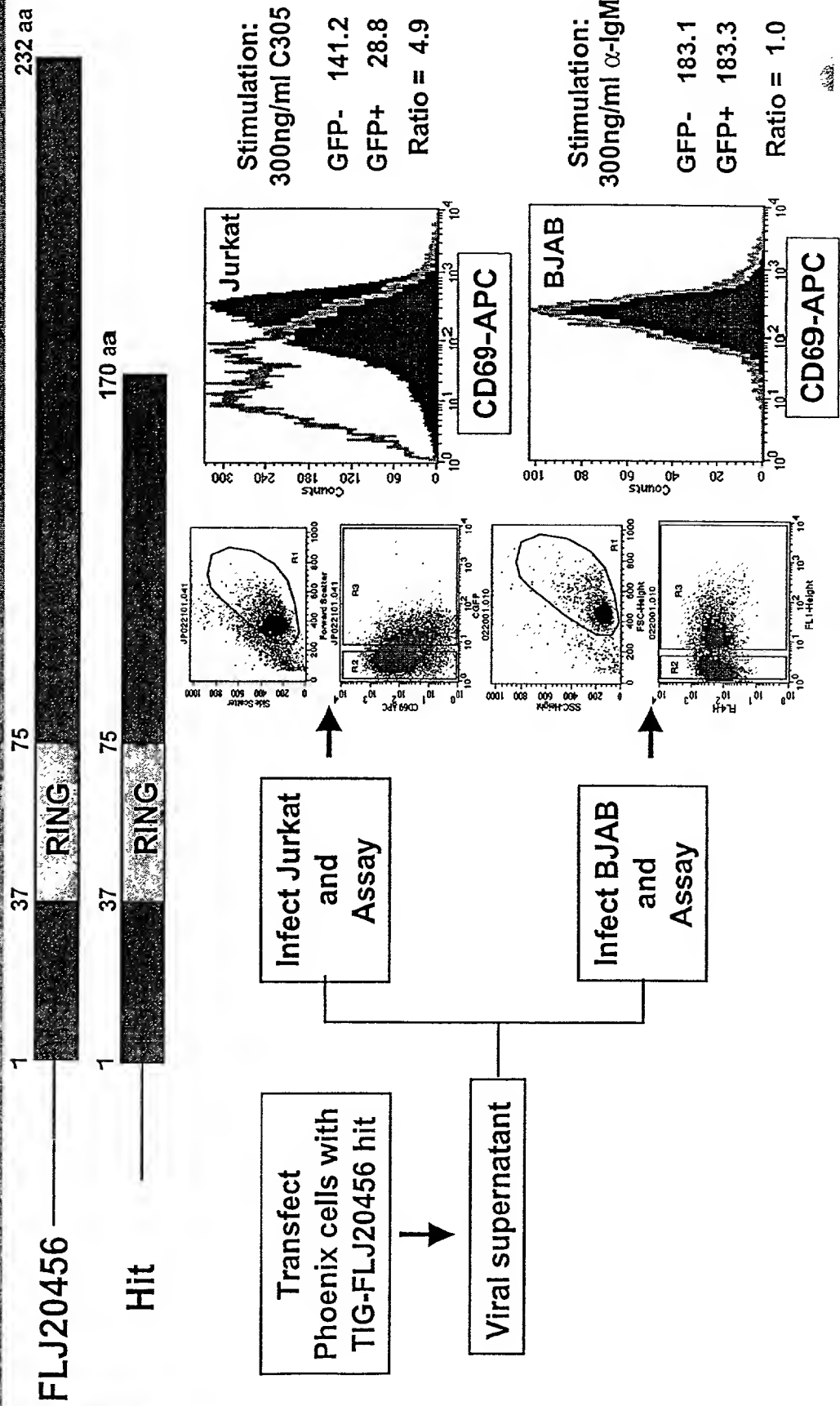
JurkatN 32H

300ng/ml C305
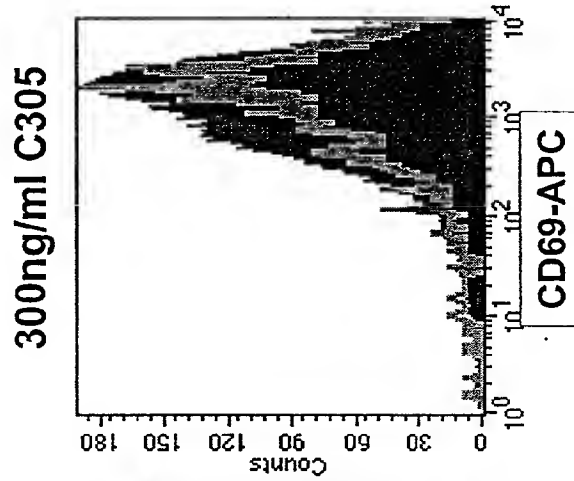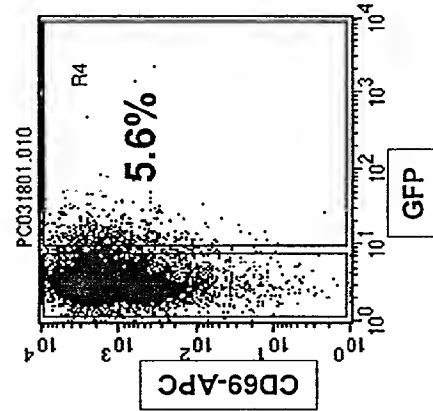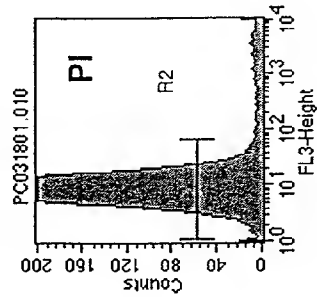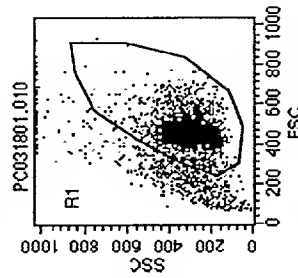
GFP-   1070.5

GFP+   1219.9

Ratio = 0.88

CD69-APC

5.6%   R4   GFP

CD69-APC

PI   R2   FL3-Height

FSC / SSC   R1

Figure 4

FLJ20456 Hit Specifically Inhibited T Cell Activation but not B Cell Activation

Figure 5

FLJ20456 Is Strongly Expressed in Lymphoid and Hematopoietic Organs

Probe: FLJ20456 Hit

- FLJ20456 is expressed in multiple tissues
- The strongest expression is in PBL, Liver and Spleen

Figure 6

TRAC1 is Predominantly Expressed in Resting T lymphocytes
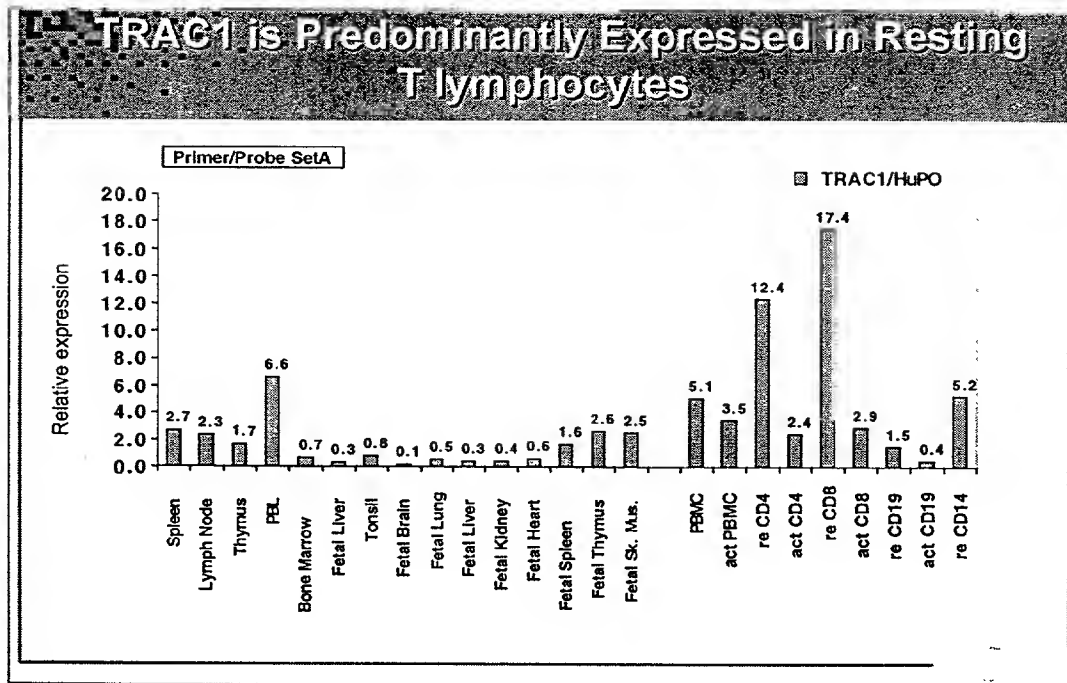
Figure 7

FLJ20456 Sequence is Most Similar to Two Sequences : Znf313 and STRIN

Ring domain

Consensus #1 . . . . F . C . VC . EV . . . PV . . . . C . HVFC . . C . . . .

FLJ20456.pep MGSVLSTDSGKSAPASATARALERRRDPELFVTSFDCAVCLEVIHQPVE-TRCGHVFCRSCIAISLKN 67
znf313.pep - - - - MAAQQRDCGGAAQLAGPAAEADELGRFICPVCLEVYEKPVQV-PCGHVFCSACLQECLKP 59
STRIN.pep - - - - - MAEDLSAATSYTEDDFYCQEVLKTPVRTACQHVFCEKCFLTAMRE 49

Consensus #1 . . . . C . . . CR . . . . A . . . . C . . . . R . H . . C . KY . . Y . . . . . .

FLJ20456.pep NKWTCPYCRAYLPSE - - - GVPATDVAKRMKSEYKNCAECDTLVCLSEMRAHIRTCQKYIDKYGPLQE 131
znf313.pep KKIPVCGVCRSALAP - - - GVRAVELERQIEISTETSCHGCRKNFFLSKIRSHVATCSKYQN-YIMEGV 121
STRIN.pep SGAHCBLCRGNVTRRERACPERALDLENIMRKFSGSCRCCAKQIKFYRMRHHYKSCKKYQDEYGVSSI 117

Consensus #1 . . . . CP . C . . . . L . HC . . H . . . . . .

FLJ20456.pep LEETAAR- - - - - - - - - - - - - - - - - - CVCPFCORELYE-DSLLDHCITHRSERR 166
znf313.pep KATIKDASLQPRNVPNRY- - - - - - - - - - - TFPCPYCPEKNFDQEGIVEHCKLFHSTDTK 169
STRIN.pep VPNFQISQDSVGNSNRSETSTSDNTETYQENTSSSGHPTFKCPLCQESNETRQRLLDHCNSNHLFQIV 185

Consensus #1 . V . CP . C . . . . P . . . P . . . N . . H . . . . . Y . . F . . . EE . . . . S . . . .

FLJ20456.pep PVFCPLCFLRLIPDENPSSFISGNLIRHLQVSHTLFYDDFIDFNIEEALIRRVLDRSLLEYVNHSNTT- 233
znf313.pep SVVCPICAISMPWGDPNYRSANFREHIQRRHRFSYDTEVDYDVDEEDMMNQVLQRSIIDQ. 229
STRIN.pep PVTCPICVSLPWGDPSQITRNEVSHLNQRRQEDYGEFVNLQLDEETQYQTAVEESFQVNI. 246

Percent Identity

				1	2	3	
divergence	1				26.6	22.3	1 FLJ20456.pep
	2	130.4				27.9	2 znf313.pep
	3	140.9	134.7				3 STRIN.pep
		1	2	3			

- All three sequences are human
- Murine sequences are not shown

Figure 8

Alignment of RING Domain Sequences from Various Human Proteins

Consensus #1 . . . . . C . . . C . . . . C . . . . C . H . C C

FLJ20456.Ring	VTSFDCAVCLEVIHQPVRTR-CGHVFCRSCIATSL--KNNKWTCPYCRAYLP-S	50
znf313.Rdng	LGRFTCPVCLEVYEKPVQVP-CGHVFCSACLQECL--KPKKPVCGVCRSALA-P	50
STRIN.Rdng	EDDFYCPVCQEVLKTPVRTTA-CQHVFCRKCFLTAM--RESGAHCPLCRGNV-T	50
TRAF6.Ring	ESKYECPHCLMALREAVQTP-CGHRFCKACIIKSI--RDAGHKCPV-DNEILLE	50
c-Cbl.Ring	STFQLCKICAEN-DKDVKIEP-CGHLMCTSCLTSW-Q-QESEGQGCPFCRCEI-KG	50
BRCA1.Ring	--LECPHCLELIKEPVSTK-CDHIFCKFCMLKLLNQ-KKGPSQCPLCKNDITKR	50
BAR.Ring	VSEFSCHCCYDIEVNPT-TLNCGHSFCRHCLALWA--SSKKTECPCREK-IWE	50
RAG1.Ring	VKSISCQICEHILADPVETN-CKHVFCRVCILRCL--KVMGSYCPSCRYPCF-P	50

RING

FLJ20456

TRAF6   CB   MATH

Cbl   CB   TB

BRCA1   CB   BRCT   BRCT

BARD1   CB   SAM

RAG1   CB

Figure 9

RING finger vs. Zinc finger proteins

Ring-HC: C_3HC_4 = Cys in position 5
Ring H2: C_3H2C_3 = His in position 5

- Ring finger domains have a conserved pattern of Cys and His residues that co-ordinate two zinc atoms to form a cross-brace structure
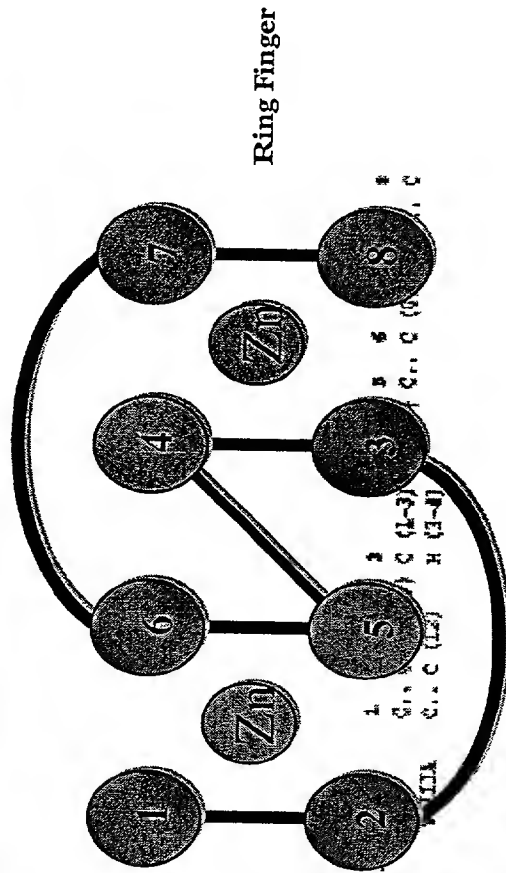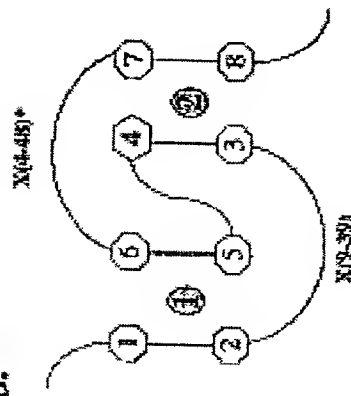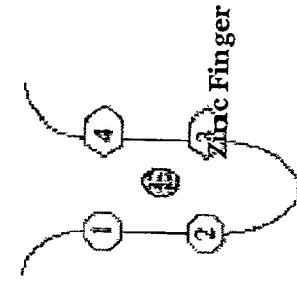
- Ring fingers are structurally distinct from zinc fingers

Figure 10

Ubiquitin Pathway Components

- E1: ubiquitin-activating enzyme, with a major isoform that may work broadly

- E2: ubiquitin-conjugating enzyme, a class of ~14 enzymes, interacts with E3

- E3: ubiquitin ligases, a broad and growing group of activities that promote addition of ubiquitin to specific proteins

- Proteasome-a 26S complex containing a 19S lid and base that mediates ATP- and ubiquitin-chain-dependent binding of substrates and a 20S catalytic core with three known proteolytic activities.

10 A

10 B

Enzymology of Ubiquitynation

Ub = ubiquitin	E2 = ubiquitin conjugating enzyme
E1 = ubiquitin activating enzyme	E3 = ubiquitin ligase

Figure 11α

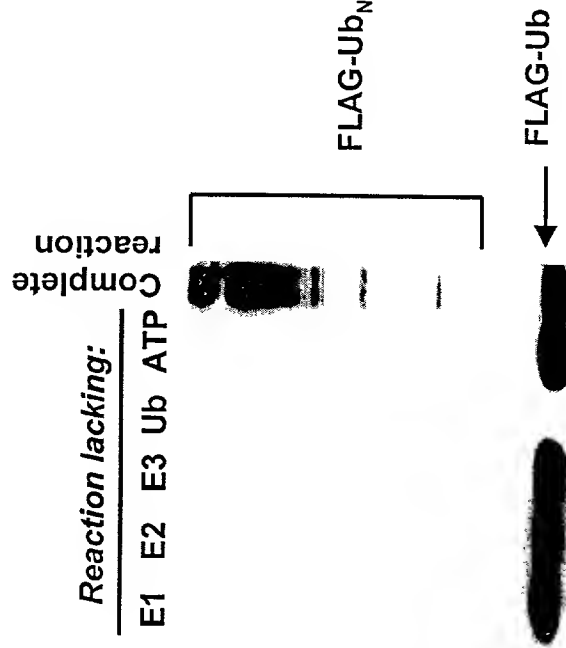A Reconstituted, Substrate-independent Assay for Studying Ligase Catalysis

Reaction Components

E1:

E2 (UbcH5): GST-fusion (cleaved), E. coli

E3 (Ring/cullin): His-tagged, coexpressed, baculovirus
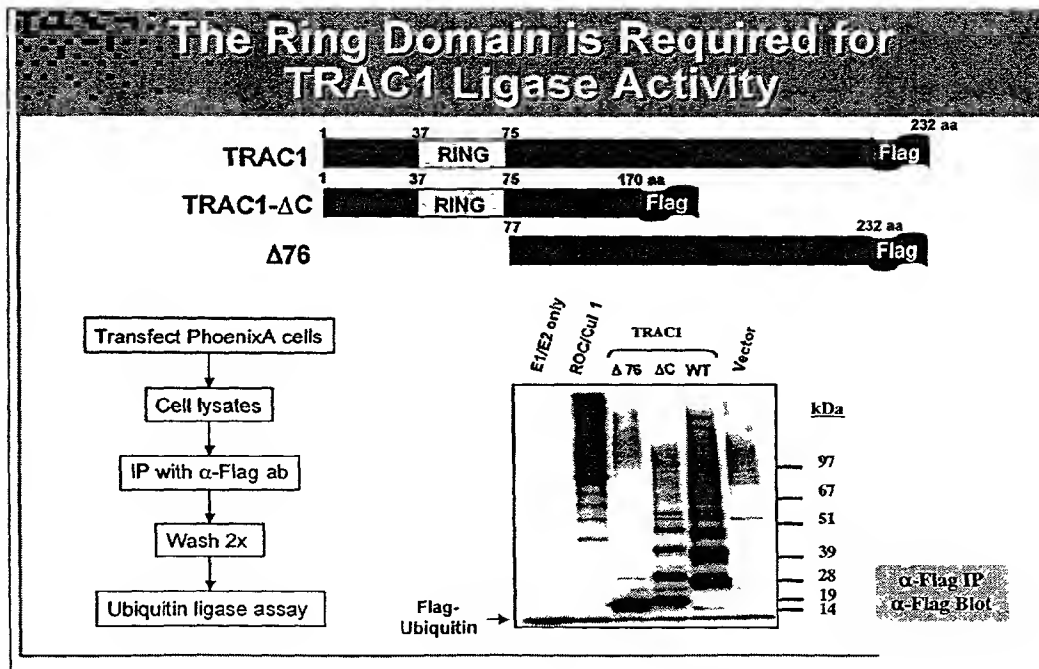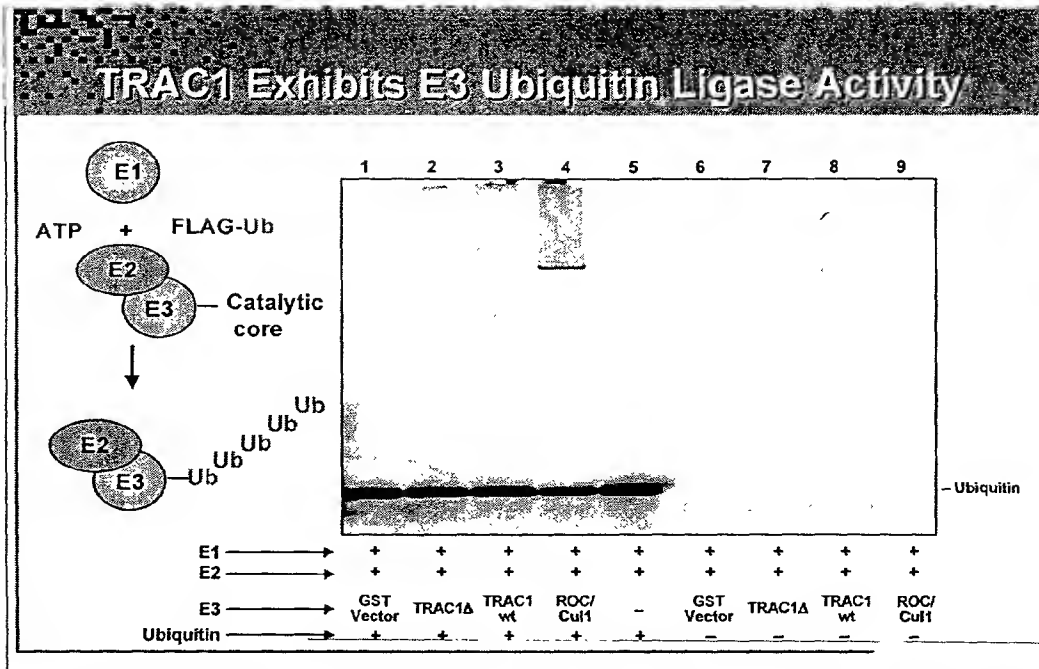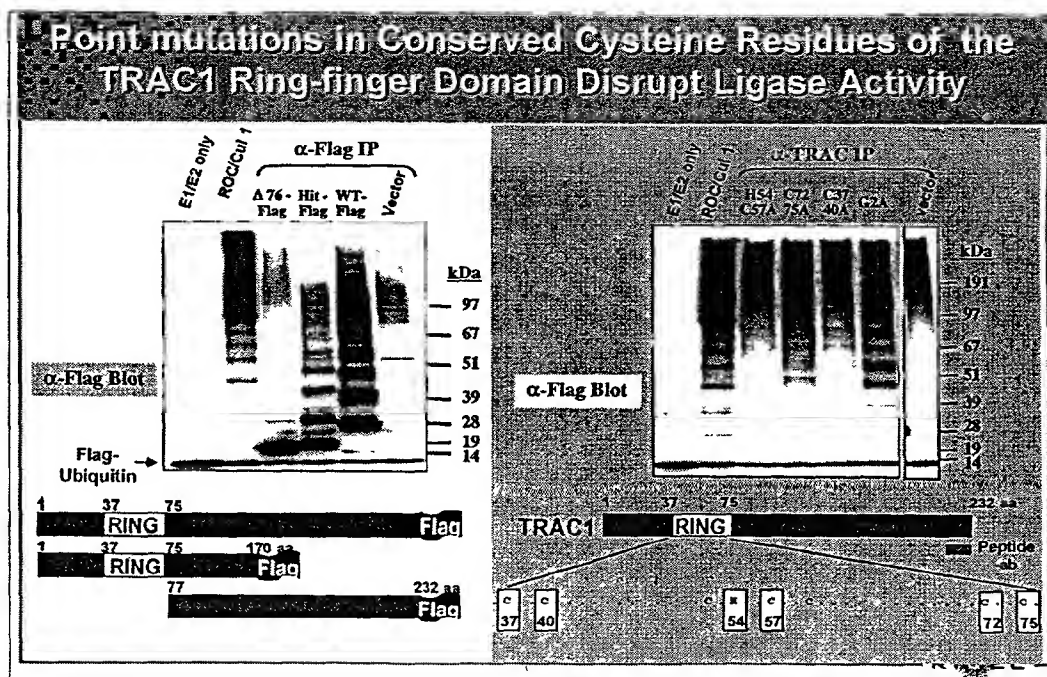
Ubiquitin: FLAG-tagged, E. coli

Reaction lacking:

E1 E2 E3 Ub ATP

Complete reaction

FLAG-Ub_N

FLAG-Ub

FLAG-Ub

ATP + FLAG-Ub

E1

Roc1

Cul1 E2 — Catalytic core

Roc1

Cul1 E2 — UbUbUbUbUbUbUb

The substrate-independent reaction has the same catalytic properties and requirements for Roc1/Cul1 as the substrate-dependent reaction

Figure 11 B

Bacterially-expressed TRAC1 for Ligase Activity Assay

Figure 12

TRAC1 Exhibits E3 Ubiquitin Ligase Activity

	1	2	3	4	5	6	7	8	9
E1	+	+	+	+	+	+	+	+	+
E2	+	+	+	+	+	+	+	+	+
E3	GST Vector	TRAC1Δ	TRAC1 wt	ROC/Cul1	–	GST Vector	TRAC1Δ	TRAC1 wt	ROC/Cul1
Ubiquitin	+	+	+	+	+	–	–	–	–

12A

The Ring Domain is Required for TRAC1 Ligase Activity

12B

Figure 13

Point mutations in Conserved Cysteine Residues of the TRAC1 Ring finger Domain

13A

Point mutations in Conserved Cysteine Residues of the TRAC1 Ring-finger Domain Disrupt Ligase Activity

13B

Figure 14

Expression of TRAC-1 mRNA is ~8 fold higher in PBMC than in Jurkat cells

Figure 15

C-terminal Truncated TRAC1 Blocks TCR-induced Ca²⁺ Influx

Figure 16

An Intact TRAC1 Ring domain is Required for Inhibition of α-TCR-Induced CD69 Up-regulatio

CD69-APC (Geometric Mean)

Legend:
- Unsti. GFP-
- Unsti. GFP+
- C305 GFP-
- C305 GFP+

X-axis categories: Vector, WT, H54A, H54C, Hit, Hit-H54A, Hit-C75A, Hit- C37,40A, Hit- H54C57A, Hit- C72,75A, ?76
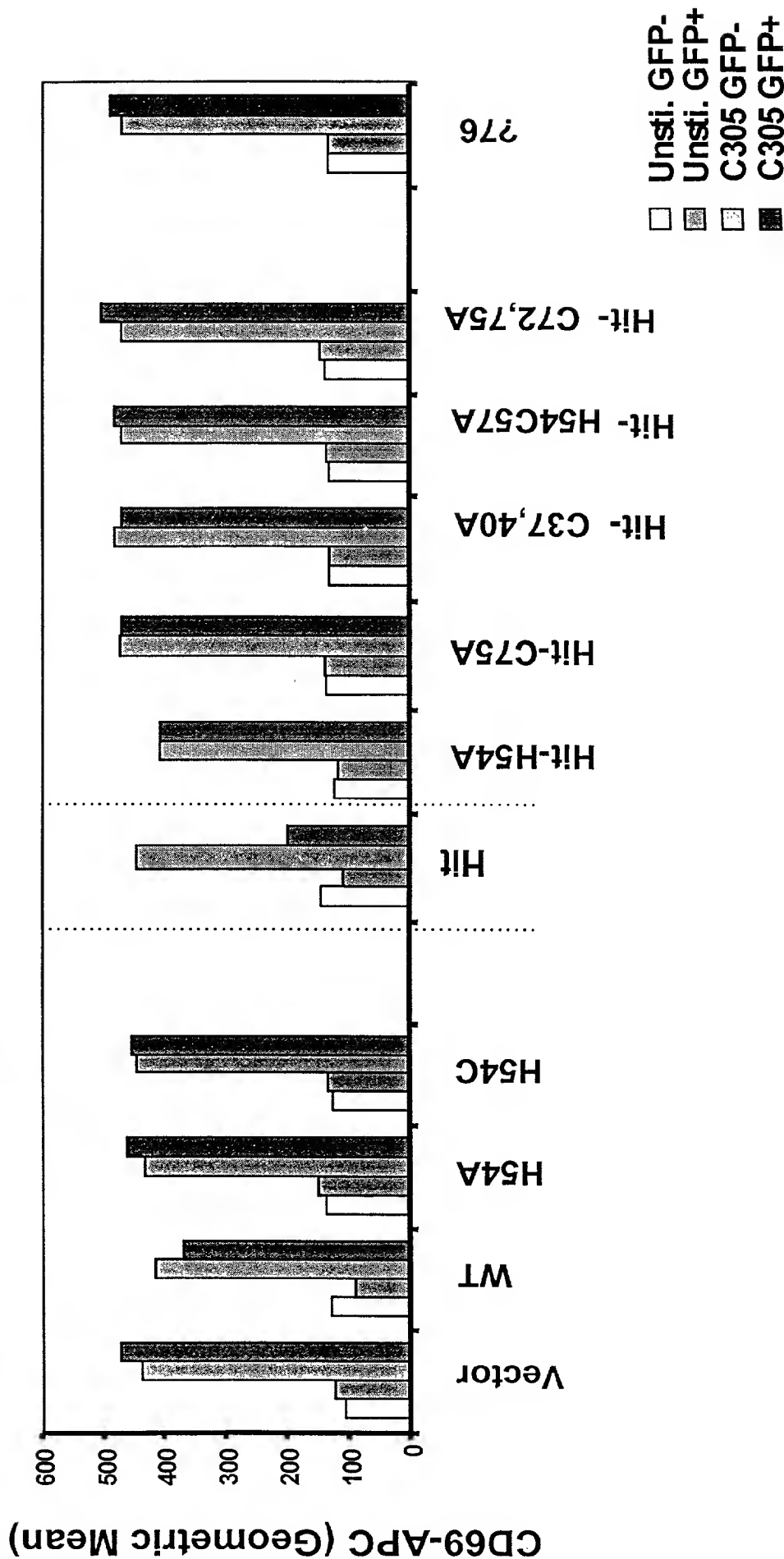
Figure 17

Summary of Functional Effects by Different TRAC-1 constructs

Figure 18

Transiently Transfected TRAC1 Protein Binds to Ubiquitin-Conjugating Enzymes (E2s) UbcH7 and UbcH5 *in vitro*

Cellular Lysate Transfected w/
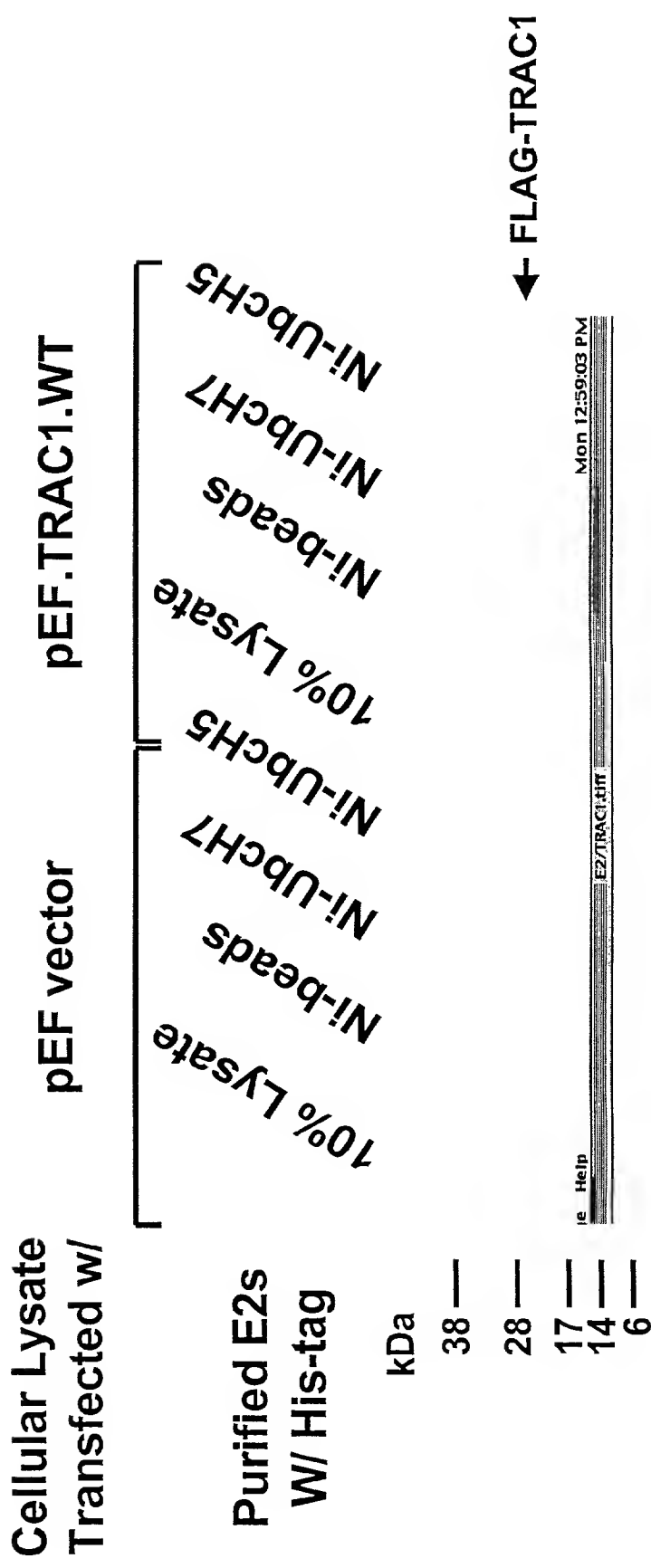
pEF vector   pEF.TRAC1.WT

Purified E2s W/ His-tag

pEF.TRAC1.WT
Ni-UbcH5
Ni-UbcH7
Ni-beads
10% Lysate

pEF vector
Ni-UbcH5
Ni-UbcH7
Ni-beads
10% Lysate

kDa
38 —
28 —
17 —
14 —
6 —

← FLAG-TRAC1

Mon 12:59:03 PM
E2/TRAC1.tiff
le   Help

Figure 19